Mutations in DNAH5 account for only 15% of a non-preselected cohort of patients with primary ciliary dyskinesia

M Failly, L Bartoloni, A Letourneau, A Munoz, E Falconnet, C Rossier, M M de Santi, F Santamaria, O Sacco, C D DeLozier-Blanchet, R Lazor and J-L Blouin

J. Med. Genet. 2009;46;281-286
doi:10.1136/jmg.2008.061176

Mutations in *DNAH5* account for only 15% of a non-preselected cohort of patients with primary ciliary dyskinesia

M Failly,[1] L Bartoloni,[1] A Letourneau,[1] A Munoz,[1] E Falconnet,[1] C Rossier,[1] M M de Santi,[2] F Santamaria,[3] O Sacco,[4] C D DeLozier-Blanchet,[1] R Lazor,[5,6] J-L Blouin[1,7]

► Additional tables and figure are published online only at http://jmg.bmj.com/content/vol46/issue4

[1] Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva, Switzerland; [2] Department of Human Pathology and Oncology, University of Siena, Italy; [3] Department of Pediatrics, Federico II University, Napoli, Italy; [4] UO Pneumologia IRCCS G. Caslini, Genova, Italy; [5] Department of Respiratory Medicine, University Hospital of Bern, on behalf of the Swiss Group for Interstitial and Orphan Lung Diseases (SIOLD), Bern, Switzerland; [6] Reference Center for Orphan Pulmonary Diseases, Louis Pradel University Hospital, Lyon, France; [7] Genetic Medicine, University Hospitals of Geneva, Geneva, Switzerland

Correspondence to: Dr J-L Blouin, Genetic Medicine and Development, University Medical Center, Rue Michel Servet 1, CH-1211 Geneva 4, Switzerland; jean-louis.blouin@medecine.unige.ch

Received 19 June 2008
Revised 3 September 2008
Accepted 11 September 2008

ABSTRACT

Background: Primary ciliary dyskinesia (PCD) is characterised by recurrent infections of the upper respiratory airways (nose, bronchi, and frontal sinuses) and randomisation of left–right body asymmetry. To date, PCD is mainly described with autosomal recessive inheritance and mutations have been found in five genes: the dynein arm protein subunits *DNAI1*, *DNAH5* and *DNAH11*, the kinase *TXNDC3*, and the X-linked retinitis pigmentosa GTPase regulator *RPGR*.

Methods: We screened 89 unrelated individuals with PCD for mutations in the coding and splice site regions of the gene *DNAH5* by denaturing high performance liquid chromatography (DHPLC) and sequencing. Patients were mainly of European origin and were recruited without any phenotypic preselection.

Results: We identified 18 novel (nonsense, splicing, small deletion and missense) and six previously described mutations. Interestingly, these *DNAH5* mutations were mainly associated with outer + inner dyneins arm ultrastructural defects (50%).

Conclusion: Overall, mutations on both alleles of *DNAH5* were identified in 15% of our clinically heterogeneous cohort of patients. Although genetic alterations remain to be identified in most patients, *DNAH5* is to date the main PCD gene.

Primary ciliary dyskinesia (PCD) (OMIM 242650) or immotile cilia syndrome is a clinically heterogeneous disease that affects all ciliated cells in 1 in 20 000 live births (1/12 500 to 1/30 000).[1] Defective ciliary function in the respiratory system impairing mucociliary clearance results in chronic sinusitis and upper respiratory tract infections, leading later to bronchiectasis and nasal polyps.[2] When PCD is associated with situs inversus, which occurs in 50% of patients, the condition is called Kartagener syndrome (KS) (OMIM 244400).[3]

The most common ultrastructural abnormalities observed in PCD involve the outer dynein arm (ODA) and/or inner dynein arm (IDA), which can be absent or shorter. Dynein arms are multisubunit complexes composed of several light, intermediate and heavy chains, encoded by distinct loci dispersed throughout the genome. Other abnormalities may include microtubule transpositions and radial spoke defects.[4]

PCD, which generally follows an autosomal recessive inheritance pattern, is also highly genetically heterogeneous as first suggested by linkage studies.[5–8] So far, five PCD-causing genes have been identified including the two heavy axonemal chain dyneins *DNAH5* and *DNAH11*, and the intermediate chain axonemal dynein *DNAI1*.[9–15] Another candidate gene, encoding thioredoxin-nucleoside diphosphate kinase *TXNDC3*, was recently characterised in a few patients.[16] An additional gene, *RPGR*, is responsible for an X-linked form associated with retinis pigmentosa in one family.[17]

Previous studies identified mutations in either *DNAI1* or *DNAH5* in approximately 38% of a cohort of mostly preselected PCD/KS patients. Of the five currently known genes associated with PCD, the *DNAI1* gene has been the object of most studies,[9–12 17 18] although mutations in the coding or splicing sequences have been found in only 10% of mostly preselected PCD patients and more recently in only 2% in a cohort of unselected individuals.[18] In the current study we assessed the frequency of mutations in the *DNAH5* gene in a large cohort of PCD patients without any preselection based on axoneme ultrastructure and/or genotype. *DNAH5* had been identified using homozygosity mapping and candidate gene approach.[19] This 79 exon gene (with one alternative first exon) codes for a heavy chain that localises in the outer dynein arm and is the homolog of the dynein γ-heavy chain of *Chamydomonas reinhardtii*. Previous studies found *DNAH5* mutations in 28% of the analysed families with PCD,[15] and a total of 42 mutations have been reported (table 1).[14 15 20] Recessive homozygous mutations in *Dnahc5*, the mouse ortholog of *DNAH5*, confirmed the involvement of this gene in PCD. Homozygous embryos had normal organ situs in 25%, situs inversus totalis in 35%, and heterotaxy in 40%. Embryos with heterotaxy had complex structural heart defects.[21]

The rationale to assess the *DNAH5* gene in an independent cohort of patients included: (1) the DNAH5 protein is localised within the ODA of the ciliary axoneme arm which is often (60–70%) absent or shortened in PCD patients[22 23]; (2) to date, *DNAH5* mutations represent the most common known cause of PCD (28%). However, prevalence of *DNAH5* mutations found in PCD might have been biased since patients were mostly recruited through preselection for linkage to the *DNAH5* locus[15] or mainly for ODA defect.[14]

PATIENTS AND METHODS

Patients

Case recruitment and selection

We collected blood samples (n = 108) from patients and families (unaffected parents and affected/unaffected siblings). Patients originated from

Table 1 Summary of the mutations already published for the *DNAH5* gene

	DNA change	Protein change	Ref
Exon			
3	c.232C>T	p.R78X	15
3	c.252T>G	p.Y84X	15
7	c.832delG	p.A278RfsX27	15
11	c.1426_1427del	p.F476SfsX26	15
12	c.1627C>T	p.Q543X	15
13	c.1730G>C*	p.N549_R577delfsX5	15
14	c.1828C>T	p.Q610X	14
25	c.3905del	p.L1302RfsX19	15
28	c.4360C>T	p.R1454X	14
28	c.4361G>A	p.R1454Q	14
32	c.5130_5131insA	p.R1711TfsX36	14
32	c.5147G>T	p.R1716L	15
33	c.5281C>T	p.R1761X	15
33	c.5482C>T	p.Q1828X	15
34	c.5563_5564insA	p.I1855NfsX6	14
34	c.5599_5600insC	p.I1867PfsX35	15
36	c.6037C>T	p.R2013X	15
41	c.6791G>A	p.S2264N	15
43	c.7039G>A	p.E2347K	15
45	c.7502G>C	p.R2501P	15
48	c.7914_7915insA	p.R2639TfsX19	15
48	c.7915C>T	p.R2639X	14
49	c.8029C>T	p.R2677X	15
49	c.8167C>T	p.Q2723X	15
50	c.8314C>T	p.R2772X	15
50	c.8404C>T	p.Q2802X	15
50	c.8440_8447del	p.E2814fsX1	14
51	c.8528T>C	p.F2843S	15
53	c.8910_8911delATinsG	p.2970SfsX7	14
60	c.10226G>C	p.W3409S	15
62	c.10555G>C	p.G3519R	14
63	c.10815del	p.P3606GfsX23	15
67	c.11528C>T	p.S3843L	15
73	c.12614G>T	p.G4205V	15
77	c.13426C>T	p.R4476X	15
77	c.13458_13459insTb	p.N4487fsX1	15
77	c.13486C>T	p.R4496X	15
Intron			
17	c.2577+2T>C	splice mutation	15
27	c.4355+1G>A	splice mutation	15
74	c.12706−1G>C*	p.S4304DfsX6	25
75	c.12910−2A>T	splice mutation	15
76	c.13338+5G>A	splice mutation	15

Ref, references.
*RNA tested.

various geographical locations as described previously but were mainly Caucasian (92%).[18]

Diagnosis of PCD/KS was based on criteria previously established.[18] Among the 108 patients, inclusion criteria were fulfilled in the 89 individuals that constituted the study population (supplemental table E1) including seven families with a declared parental consanguinity. Fifty-five per cent of individuals had situs inversus (Kartagener syndrome). Ultrastructural information on electron microscopy of ciliary axonemal section was available for 74% (n = 66) of the patients. Among the patients showing dynein arms defects, 50% had a combined ODA+IDA defect, 18% had an isolated ODA defect, and 12% had an isolated IDA defect. The remaining 14% had other ultrastructural features, whereas 6% had normal ultrastructure of the ciliary axoneme.

Written informed consent was obtained from all patients, and the research protocol was approved by the ethics committee of the respective institutions.

Mutation analysis

Polymerase chain reaction amplification

Genomic DNA was isolated from peripheral blood using standard extraction procedures. The entire *DNAH5* gene was analysed including the alternative first exon and the 5′- and 3′-untranslated regions (UTR). Specific primer pairs were designed for the 80 exons at least 50 bp away from exon limits to allow polymerase chain reaction (PCR) amplification and sequence analysis of the entire exon and the flanking intronic splicing sites.

Denaturing high performance liquid chromatography

Denaturing high performance liquid chromatography (DHPLC) analysis was performed as described previously.[18] We analysed all the samples included in the cohort for all the exons of *DNAH5*, except those analysed by direct sequencing (see sequence analysis for details). Briefly, DNA heteroduplexes were produced by denaturation of PCR products at 95°C for 10 min, followed by a slow and gradual annealing of single strands from 95°C to 25°C over a 30 min period. Heteroduplexes were resolved from homoduplexes using a WAVE 3500 HT DNA DHPLC fragment analysis system and with the Navigator raw data interpretation software (Transgenomic, Omaha, Nebraska, USA).

Sequence analysis

All changes in DHPLC were further confirmed by direct sequencing (Applied Biosystems, 3130 xl Genetic Analyzer, Foster City, California, USA). In addition, all exons previously described to harbour a cluster of mutations (34, 50, 63, 76 and 77)[15] or with isolated mutations (14, 25, 28, 32, 48, 53, 62, 75)[14] were directly sequenced. The entire *DNAH5* gene was directly sequenced in patients from families in whom consanguineous marriage had been documented (n = 7), and in patients with mono-allelic mutation. Exons from homozygous regions that had been identified by single nucleotide polymorphism (SNP) genotyping were also analysed by direct sequencing in the respective patients.

Whenever a missense variant was found, the possibility that we were dealing with a non-pathological polymorphism was considered by, in the following order: (1) interrogating the SNP database (www.ncbi.nlm.nih.gov/dbSNP/); (2) verifying the amino acid conservation among species using the UCSC genome browser (www.genome.cse.ucsc.edu/); (3) screening for the variant in at least 160 chromosomes of same ethnic origin by direct sequencing or minisequencing (Pyrosequencing, Biotage AB, Uppsala, Sweden). All identified mutations were confirmed by repeating sequencing on the original DNA sample source tube.

SNP analysis

SNP analysis on the six linkage disequilibrium (LD) blocks encompassing gene *DNAH5* in Caucasians (www.hapmap.org) was performed as described previously[18] by minisequencing (Pyrosequencing, Biotage AB, Uppsala, Sweden). The following SNPs were genotyped: rs10513151, rs6860899, rs17278234, rs4702001 and rs4702002 (LD block 1); rs10057950, rs6859484, rs1445823, rs7721634 and rs962138 (LD block 2); rs11748811, rs13170062, rs1900162, rs17275618 and rs6554820 (LD block 3);

rs980897, rs795542, rs924630, rs1596790 and rs795540 (LD block 4); rs6554810, rs4701984, rs6554811, rs6867796 and rs4351149 (LD block 5); rs3734110, rs6862469, rs10513151, rs1502046 and rs2896104 (LD block 6). Briefly, PCRs were performed using 50 ng of template DNA and checked for size and yield on a 2% agarose gel. Amplicons were purified and minisequenced using 30 pmol of sequencing primer per reaction under standard conditions according to the manufacturer's instructions (Pyrosequencing, Biotage AB, Uppsala, Sweden) in a Pyrosequencing PSQ HS 96 System. Data were captured using PSQ HS 96 SNP software (Pyrosequencing, Biotage AB, Uppsala, Sweden).

In vitro splicing assay

When RNA was not available, an in vitro splicing assay was used to characterise the effect of the splicing mutation on the DNAH5. As described previously,[24] the wild-type and the mutated constructs were generated by PCR amplification using oligonucleotides in *DNAH5* introns 6–7 and 9–10 (6195-F: ATTGGAAGCA TGGAATACGC/6195-R: TTGAACTGA GCCAATGTGGT), 23–24 and 26–27 (170-F: TGGGTTAGAG GGCAATAAGC/170-R: GGGCCCTCTAT CTTACAAAGAA) and 33–34 and 35–36 (167-F: AGGAAACAATGAGAA ACGTGAC/167-R: AAAGAGCCTATAAACCCTAAGAGAC). The PCR fragments were cloned into the pcDNA3.1/V5-His TOPO-TA mammalian expression vector (Invitrogen, San Diego, California, USA), and their integrity verified by sequencing. The constructs were transfected into HEK-293T cell line using Lipofectamine 2000 (Life Technologies, Basel, Switzerland). Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, California, USA), and reverse transcriptase (RT)-PCR was performed (Invitrogen, San Diego, California, USA). The primers used for the PCR as well as for sequencing were as follows: Exon 7F: GAGCTGGAGCACTGGAAAAA and Exon 9R: CAGAGATG TGATCTTCTCAGAGGT. Exon 24F: ACCGGAGTGAGAT GGAAAAC and Exon 26R: CGCTAGCC-ATTGGACCAT. Exon 34F: CACCACGAGGGATCTGAGTT and Exon 35R: CCTGTCAGTGCAGCCTAAAA.

RESULTS

Mutational analysis of DNAH5

We carried out mutation analysis using a combination of DHPLC and sequencing on a cohort of 89 patients with confirmed PCD/KS, recruited without preselection. Analysis of all coding exons, including splice site junction and UTR regions, revealed 18 novel *DNAH5* mutations in 16 patients in addition to six previously reported mutations that we also identified in six patients.[15] We observed missense (43%), nonsense (40%), indels, and splice site mutations (17%) (table 2 and supplemental table E2 for clinical details).

Localisation of these mutations within the specific domains of DNAH5 is depicted in supplemental fig E1. We identified two mutations in 15% of the patients, while 18% had at least one mutated allele. The 18 novel variants included 10 missenses (p.I370F, p.F540L, p.N549D, p.D556G, p.R1716W, p.R2630W, p.V2829F, p.R2833G, p.E3455H and p.R3539C) found in seven patients (tables 2 and 3). Four missenses—p.I370F, p.F540L, p.N549D, p.D556G—were localised within the N-terminal region 1 domain (DHC_N1) of DNAH5 which is known to form dimers with other heavy chains, and with intermediate chain–light chain dynein complexes involved in a basal cargo binding unit.[25] The functional importance of this domain has been demonstrated recently in a mouse model in which a targeted homozygous deletion resulted in PCD as well as structural heart defects in 40% of the embryos.[21]

A substitution at amino acid 1716 was previously reported as a pathogenic alteration.[15] The novel substitution p.R1716W, which is not conservative, may similarly affect the function of the protein as described by the other study. The following changes that cause missenses within conserved functional domains of the protein—p.R2630W lying within the AAA3 domain (ATPases associated diverse cellular activities)[26]; p.V2829F and p.R2829G lying between the AAA3 and AAA4 domains; p.E3455H located in the microtubule binding site (MTB); p.R3539C localised between the MTB and the P5 loop—are strongly suspected to be disease causing mutations. Finally, the previously identified frameshift mutation p.D4398EfsX16 was found in two patients, either as a compound heterozygote

Table 2 *DNAH5* mutations and phenotypes of primary ciliary dyskinesia patients

Patient	Geographic origin	Location	DNA change	Protein change
Homozygous				
169	Italy	[**34**]+[**34**]	c.[**5647C>T**]+[**5647C>T**]	p. [**R1883X**]+[**R1883X**]
Compound				
170	Italy	[**6**]+[**25, Intron 26**]	c.[**670C>T**]+[**3876_4053+158del**]	p. [**R224X**]+[**E1279-K1351del**]
6195	USA	[**Intron 8**]+[**9**]	c.[**1089+1G>A**]+[**1108A>T**]	p. [**splice***]+[**I370F**]
8149	Switzerland	[**13**]+[**13**]	c.[**1645A>G**]+[**1667A>G**]	p. [**N549D**]+[**D556G**]
8145	Switzerland	[**27**]+[**61**]	c.[**4348C>T**]+[**10365G>C**]	p. [**Q1450X**]+[**Q3455H**]
8171	Switzerland	[**27**]+[**72**]	c.[**4348C>T**]+[**12397G>T**]	p. [**Q1450X**]+[**E4133X**]
8182	Switzerland	[33]+[**72**]	c.[5281C>T]+[**12397G>T**]	p. [R1761X]+[**E4133X**]
8143	Switzerland	[**34**]+[49]	c.[**5557A>T**]+[8029C>T]	p. [**K1853X**]+[R2677X]
167	Italy	[**Intron 34**]+[**61**]	c.[**5710-2A>G**]+[**10365G>C**]	p. [**C1904-K1909del**]+[**Q3455H**]
8177	Switzerland	[41]+[76]	c.[6791G>A]+[13194_13197del]	p. [S2264N]+[D4398EfsX16]
6191	USA	[**12**]+[45]+[**63**]	c.[**1619T>C**]+[7502G>C]+[**10615C>T**]	p. [**F540L**]+[R2501P]+[**R3539C**]
6127	Switzerland	[**51**]+[77]	c.[**8485G>T**]+[13486C>T]	p. [**V2829F**]+[R4496X]
8131	Switzerland	[**51**]+[77]	c.[**8497C>G**]+[13486C>T]	p. [**R2833G**]+[R4496X]
Heterozygous				
206	Italy	[**32**]+[nd]	c.[**5146C>T**]+[nd]	p. [**R1716W**]+[nd]
8103	Switzerland	[**48**]+[nd]	c.[**7888A>T**]+[nd]	p. [**R2630W**]+[nd]
6476	Switzerland	[76]+[nd]	c.[13194_13197del]+[nd]	p. [D4398EfsX16]+[nd]

nd, not determined.
Novel mutations are depicted in bold.
*Two different transcripts, leading to p.T326-P363del and p.T326VfsX25, were found for the c.1089+1G>A mutation.

Table 3 Missense variants found in this study by direct sequence analysis

Base change	Amino acid change	Exon	Frequency		Conservation	DNA variation
			Control	PCD		
c.1108A>T	p.I370F	9	0/170	1/178	Yes	Novel mutation
c.1619T>C	p.F540L	12	0/160	1/178	Yes	Novel mutation
c.1645A>G	p.N549D	13	0/170	1/178	Yes	Novel mutation
c.1667A>G	p.D556G	13	0/160	1/178	Yes	Novel mutation
c.2253C>A	p.N751K	15	2/170	4/178	Yes	New SNP
c.5146C>T	p.R1716W	32	0/180	1/178	Yes	Novel mutation
c.7888A>T	p.R2630W	48	0/170	1/178	Yes	Novel mutation
c.8485G>T	p.V2829F	51	0/160	1/178	Yes	Novel mutation
c.8497C>G	p.R2833G	51	0/170	1/178	Yes	Novel mutation
c.10365G>C	p.Q3455H	61	0/166	2/178	Yes	Novel mutation
c.10615C>T	p.R3539C	63	0/170	1/178	Yes	Novel mutation

PCD, primary ciliary dyskinesia; SNP, single nucleotide polymorphism.

with the known missense p.S2264N mutation (patient 8177), or as heterozygote (mono-allelic mutation) in patient 6476. The previously described missense mutation p.R2501P was found in one patient (6191) who carries two other novel missense mutations (p.F540L and p.R3539C).[15]

We have also identified five novel nonsense mutations (p.R224X, p.Q1450X, p.K1853X, p.R1883X and p.E4133X) in five patients and the previously described nonsenses p.R1761X, p.R2677X and p.R4496X were found in three other patients.[15] Of the 16 patients in whom *DNAH5* mutations were found in this study, three patients had mono-allelic mutations (table 2).

In order to verify that any homozygous mutations were not missed because of the detection technical limitations of the DHPLC assay, we genotyped a series of SNPs in the six linkage disequilibrium blocks (LD) that encompass the whole length of the *DNAH5* gene (according to HapMap study in CEU sample, www.hapmap.org). We genotyped patients who could carry such a hidden homozygous mutation because they (1) were born from consanguineous marriages, or (2) had no identified heterozygous variants within the region(s).

As previously described,[18] a total of 30 informative SNPs (n = 5 per LD block) were selected for genotyping (data not shown). We then directly sequenced all the exons that belonged to LD blocks in which genotyping was showing total homozygosity. None of the genotyped patients showed heterozygosity in all six LD blocks. Furthermore, individuals (total n = 72) without heterozygosity in one or more LD blocks (n = 16 for block 1, n = 31 for block 2, n = 28 for block 3, n = 15 for block 4, n = 22 for block 5, and n = 21 for block 6) were directly sequenced for the respective exons of *DNAH5*. No additional mutations were detected.

Characterisation of three novel splice site mutations

In three patients (167, 170 and 6195), we detected three novel heterozygous variants (c.5710-2A>G, c.3876_4053+158del, and c.1089+1G>A, respectively) predicting altered splicing of their respective exons and subsequent aberrant transcripts of *DNAH5*. To test whether these nucleotide changes could alter the sequence of mature RNA, in vitro splicing assay of the mutant and wild type (WT) constructs in subsequent HEK-293T cells were performed (fig 1, supplemental table E3).

Variant c.5710-2A>G resulted in a PCR fragment of 220 bp instead of the 238 bp observed in WT (fig 1-A1). The sequence of this 220 bp transcript cDNA revealed that the regular acceptor splicing site was not recognised, but was replaced by a cryptic splicing site within the following exon. This results in an in-frame deletion skipping the beginning of exon 35 (p.C1904-K1909del) (fig 1-A2, 1-A3), which might result in a deleterious functional effect on the protein.

In vitro splicing analysis of the c.3876_4053+158del mutation in patient 170 (fig 1-B1) resulted in a single fragment of 180 bp, while two products of unequal intensity were obtained in the WT situation (a weak band at 180 bp and a stronger one at 399 bp) (fig 1-B2). Sequencing of the 180 bp fragment resolved a p.E1279-K1351del, while the 399 bp was confirmed as the WT product (fig 1-B3). The relatively lower intensities of the 180 bp fragment amplified may reflect the low abundance of the partially skipped exon 25 of *DNAH5* in non-pathological conditions.

Two different outcomes resulted from the c.1089+1G>A mutation splicing assay analysis in patient 6195 with abnormal transcripts of equal intensity, while the wild-type transcript was only obtained in WT construct (fig 1-C1). In mutant constructs, one transcript (the shorter) revealed a deletion of the entire exon 8 (219 bp), resulting in p.T326-P363del, while the other predicts a premature termination of translation secondary to disruption of the original reading frame (421 bp) (p.T326VfsX25) (fig 1-C2, C3).

DISCUSSION

Since the initial characterisation of *DNAH5*,[14] 60 mutations have been detected in gene *DNAH5* in a total of 223 families, including the ones reported here.[15] We find that *DNAH5* mutations are to date the most common of the known causes of PCD/KS with ODA±IDA defect which is partially in agreement with Hornef *et al*.[15] Our findings showed that, as suggested by Hornef *et al*, mutations in *DNAH5* are the most frequent causes of PCD in ODA, but the frequencies we found are lower.

In contrast to previous studies, our cohort was screened without any type of preselection based on subtype of phenotypes.[18] Patients included had abnormal ultrastructure and/or multiple clinical characteristics linked to PCD. This cohort is thought to be representative of PCD patients in the Caucasian population since the rate of occurrence of the various ultrastructural defects did not deviate from that in previously reported studies.[9 18]

In three out of the 16 patients carrying mutations, we could not find the second altered allele although all the exons (including the alternatively transcribed first exon), and the entire length of the 3′- and 5′- known UTR regions were directly sequenced. As described previously,[18] the second mutation in these three patients could lie in regions that were not screened such as introns, potential functional/regulatory

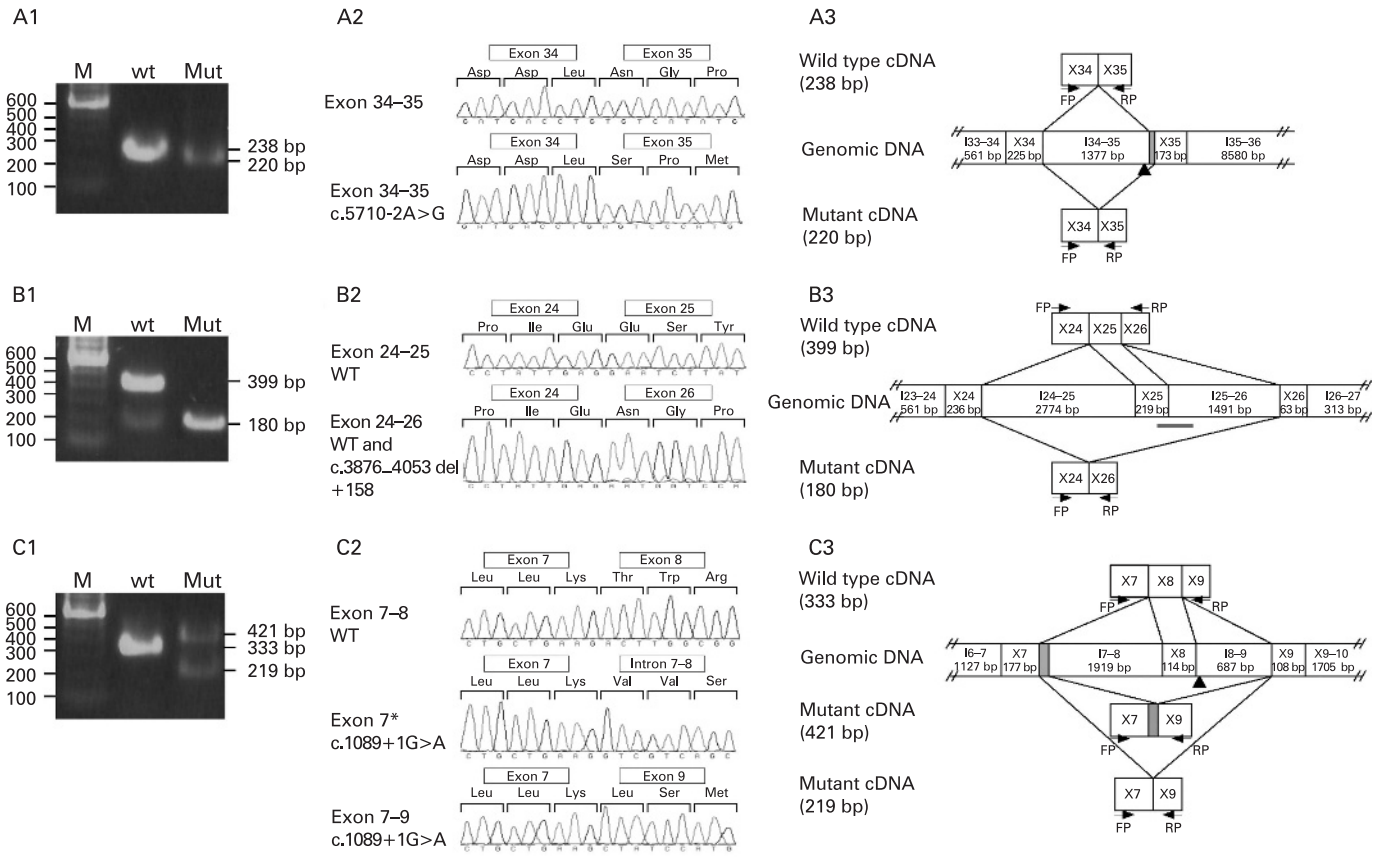

Figure 1 In vitro analysis of *DNAH5* splice site mutations. A1/B1/C1: Wild type (wt) and mutant (Mut) cDNA products of the c.5710-2A>G (A1), the c.3876_4053del+158 (B1) and c.1089+1G>A (C1) splicing mutation are shown on an agarose gel electrophoresis. M: markers. A2/B2/C2: Chromatograms showing the effect of the wild type (top) and the mutated DNA sequence (bottom) of the respective c.5710-2A>G (A2), c.3876_4053del+158 (B2) and c.1089+1G>A (C2) splicing mutations on *DNAH5* transcripts. Exon 7* represents the polymerase chain reaction (PCR) product in which the donor site of exon 7 was not recognised by the splicing machinery. A3/B3/C3: Schematic representation of the c.5710-2A>G (A3), the c.3876_4053del+158 (B3) and c.1089+1G>A (C3) mutation splicing mechanisms from the genomic DNA (middle) leading to the normal (top) and mutated (bottom) *DNAH5* transcripts. Reverse transcriptase PCR was performed using primers FP and RP located in their respective exons. Exons (X) and introns (I) are indicated with their respective sizes. Black arrowhead depicts the position of the mutation in the genomic DNA. Dark box represents the segment that is skipped after splicing (A3, C3) while the grey bar indicates the position of the deletion (B3).

sequences that are located remotely from coding regions, large deletions or insertions that are not detectable by the techniques used in this study.

Another hypothesis would be a digenic or triallelic inheritance as described in some cases of Bardet Biedl syndrome.[27] Despite the mutation screening on the *DNAI1* gene, we cannot completely rule out this hypothesis before other genes encoding for dyneins contributing to the ODA complex have been thoroughly screened for mutations. Finally, *DNAH5* may not be the gene causing PCD in these three patients with only a single mutation.

Overall in our cohort, alterations of the sequence of *DNAH5* mutations are responsible for PCD in 15% of the 89 unrelated patients analysed here. This contrasts with the study of Hornef *et al*,[15] in which *DNAH5* mutations were identified in 28% of PCD patients. Most PCD patients have ODA defects (≈60–70%),[22 28 29] and to date *DNAH5* was the most frequently mutated gene in patients with a documented ODA defect. Considering ODA defect alone as a distinct phenotype from the combined ODA+IDA, we found that 33% of our ODA patients carry a mutation in *DNAH5* which is less than expected according to the study of Hornef *et al* (49% of the patient with documented ODA defects have at least one *DNAH5* mutation).[15] In our cohort, it represents 24% of patients with ODA+IDA defect who have mutations in *DNAH5* and 27% when considering ODA ± IDA ultrastructure defect. No mutations are found in patients with EM defects different from ODA ± IDA.

All the patients studied here were also included in our previous *DNAI1* mutation analysis in a cohort of 104 individuals.[18] Taken together, 89 individuals have been screened for both the *DNAI1* and *DNAH5* genes (supplemental table E1). In 17% of all the patients we were able to identify two mutations in either *DNAI1*[18] or *DNAH5* (table 2). There were 22% of patients harbouring at least one mutant allele in either one of these two genes. Our finding deviates from a previous study in which mutations in either *DNAI1* or *DNAH5* genes represented 38% of all PCD patients.[9] Since both cohorts were mainly composed of Caucasian individuals, the inclusion of 21 consanguineous families and the preselection by linkage to *DNAH5* locus and/or ultrastructural phenotype may explain this deviation between studies. According to our results the molecular diagnostics of PCD should not rely only on *DNAI1* and *DNAH5* genes, since diagnostic sensitivity does not exceed 17%.

Most of the candidate genes for PCD—mainly heavy and intermediate chains for axonemal dynein—have been investigated with low success rates despite the large number studied.

This means that the aetiology of PCD and Kartagener syndrome remains unknown in most cases in our cohort (83%). The identification of most, if not all, genes involved in the pathogenesis of PCD, and estimation of the real impact of each of these remains necessary to advance our knowledge of the molecular basis of PCD. This is mandatory for improved diagnostic testing, finally tuned medical management, and innovative treatment of this clinically and genetically heterogeneous disease.

Acknowledgements: Authors are grateful to all the patients and their families for participation in this research study as well as Dr M Jaspers and Professor M Jorissen, Leuven, Belgium for their contribution in the analysis of the electronic microscopy analysis. The authors would also like to thank warmly the following physicians who contributed to this study by recruiting one to several patients: A Brauchlin, A Bitton, R Aebi, C Barazzone, E Bierens de Haan, G Schwarz, J-Y Berney, P-A Guerne, S Tarab, T Rochat, Geneva; P Eng, M Künzli, Aarau; H Wacker, Allschwill; W Schäppi, Andelfingen, J Hammer, M Ruthishauser, Basel; W Graf, Bethlehem; E Horak, S Schibli, HM Schöni, Davos; K Brugger, Deissenhofen; P Terrier, Delémont; B Zimmermann, Emmenbrüke; O Brändli, Faltigberg-Wald; J C Heili, Grub; D Stefanutti, La Chaux-de-Fonds; H-U Dubach, Langenthal; M Gehri, A Calame, P Marguerat, Lausanne; P Eng, Luzern; J-G Frey, Montana; C Meili, RM Kaelin, Morges; A Wallmeroth, Muttenz; B Thiévent, Porrentruy; M Bigler, Rorschach; O Schoch, J Barben, St Gallen; A Brauchlin, J Wildhaber; H Walt, Zürich; (Switzerland); BA Afzelius, Stockolm (Sweden); M Jorissen, Leuven; L van Maldergem, Loverval (Belgium); C Le Pommelet, Avignon (France); S Ala-Mello, Helsinki (Finland); G Piatti, Milano (Italy); M Wessels, Rotterdam, (Netherlands); M Armengot, Valencia (Spain); H Cox, Birmingham (UK); D Chitayat, Toronto; R Hodder, Ottawa (Canada); R Gershoni, Haifa (Israel); E Reich, New York, D Schidlow, Philadelphia (USA).

Funding: This work was supported by grants from the Swiss National Science Foundation (#3200BO-105838, to JLB), the Carvajal ProKartagener Foundation (to JLB), and the Société Académique de Genève on behalf of Fonds Dr E Rapin (to MF and JLB). LB was supported by a Marie Heim-Vögtlin grant from Swiss National Science Foundation. The Swiss Group for Interstitial and Orphan Lung Diseases was supported by the Swiss Academy of Medical Sciences, the Swiss Respiratory Society, the Swiss Pulmonary League, the Geneva Pulmonary League, Geneva University Hospitals, Bern University Hospital, the Carvajal ProKartagener Foundation, GlaxoSmithKline, Actelion and Aventis.

Competing interests: None declared.

Patient consent: Obtained.

REFERENCES

1. **Afzelius BA,** Mossberg B. Immotile-cilia syndrome (primary ciliary dyskinesia), including Kartagener syndrome. *The metabolic and molecular bases of inherited disease*, 3 ed. New York: McGraw-Hill, 1995:3943–54.
2. **Schidlow DV.** Primary ciliary dyskinesia (the immotile cilia syndrome). *Ann Allergy* 1994;**73**:457–68.
3. **Kartagener M.** Zur Pathogenese der Bronkiektasien. Bronkiektasien bei Situs viscerum inversus. *Beitr Klin Tuberk* 1933;**83**:489.
4. **Jorissen M,** Willems T, Van der Schueren B, Verbeken E. Dynein arms and spokes after ciliogenesis in cultured respiratory epithelial cells from non-PCD individuals. *Acta Otorhinolaryngol Belg* 2000;**54**:325–32.
5. **Witt M,** Wang Yf, Wang S, Sun C, Pawlik J, Rutkiewicz E, Zebrak J, Diehl SR. Exclusion of chromosome 7 for Kartagener syndrome but suggestion of linkage in families with other forms of primary ciliary dyskinesia [letter]. *Am J Hum Genet* 1999;**64**:313–18.
6. **Meeks M,** Walne A, Spiden S, Simpson H, Mussaffi-Georgy H, Hamam HD, Fehaid EL, Cheehab M, Al-Dabbagh M, Polak-Charcon S, Blau H, O'Rawe A, Mitchison HM, Gardiner RM, Chung E. A locus for primary ciliary dyskinesia maps to chromosome 19q. *J Med Genet* 2000;**37**:241–4.
7. **Geremek M,** Zietkiewicz E, Diehl SR, Alizadeh BZ, Wijmenga C, Witt M. Linkage analysis localises a Kartagener syndrome gene to a 3.5 cM region on chromosome 15q24–25. *J Med Genet* 2006;**43**:e1.
8. **Blouin JL,** Meeks M, Radhakrishna U, Sainsbury A, Gehring C, Sail GD, Bartoloni L, Dombi V, O'Rawe A, Walne A, Chung E, Afzelius BA, Armengot M, Jorissen M, Schidlow DV, van Maldergem L, Walt H, Gardiner RM, Probst D, Guerne PA, Delozier-Blanchet CD, Antonarakis SE. Primary ciliary dyskinesia: a genome-wide linkage analysis reveals extensive locus heterogeneity. *Eur J Hum Genet* 2000;**8**:109–18.
9. **Zariwala MA,** Leigh MW, Ceppa F, Kennedy MP, Noone PG, Carson JL, Hazucha MJ, Lori A, Horvath J, Olbrich H, Loges NT, Bridoux AM, Pennarun G, Duriez B, Escudier E, Mitchison HM, Chodhari R, Chung EM, Morgan LC, de Iongh RU, Rutland J, Pradal U, Omran H, Amselem S, Knowles MR. Mutations of DNAI1 in primary ciliary dyskinesia: evidence of founder effect in a common mutation. *Am J Respir Crit Care Med* 2006;**174**:858–66.
10. **Zariwala M,** Noone PG, Sannuti A, Minnix S, Zhou Z, Leigh MW, Hazucha M, Carson JL, Knowles MR. Germline mutations in an intermediate chain dynein cause primary ciliary dyskinesia. *Am J Respir Cell Mol Biol* 2001;**25**:577–83.
11. **Pennarun G,** Escudier E, Chapelin C, Bridoux AM, Cacheux V, Roger G, Clement A, Goossens M, Amselem S, Duriez B. Loss-of-function mutations in a human gene related to Chlamydomonas reinhardtii dynein IC78 result in primary ciliary dyskinesia. *Am J Hum Genet* 1999;**65**:1508–19.
12. **Guichard C,** Harricane MC, Lafitte JJ, Godard P, Zaegel M, Tack V, Lalau G, Bouvagnet P. Axonemal dynein intermediate-chain gene (dnai1) mutations result in situs inversus and primary ciliary dyskinesia (Kartagener syndrome). *Am J Hum Genet* 2001;**68**:1030–5.
13. **Bartoloni L,** Blouin JL, Pan Y, Gehrig C, Maiti AK, Scamuffa N, Rossier C, Jorissen M, Armengot M, Meeks M, Mitchison HM, Chung EM, Delozier-Blanchet CD, Craigen WJ, Antonarakis SE. Mutations in the DNAH11 (axonemal heavy chain dynein type 11) gene cause one form of situs inversus totalis and most likely primary ciliary dyskinesia. *Proc Natl Acad Sci USA* 2002;**99**:10282–6.
14. **Olbrich H,** Haffner K, Kispert A, Volkel A, Volz A, Sasmaz G, Reinhardt R, Hennig S, Lehrach H, Konietzko N, Zariwala M, Noone PG, Knowles M, Mitchison HM, Meeks M, Chung EM, Hildebrandt F, Sudbrak R, Omran H. Mutations in DNAH5 cause primary ciliary dyskinesia and randomization of left-right asymmetry. *Nat Genet* 2002;**30**:143–4.
15. **Hornef N,** Olbrich H, Horvath J, Zariwala MA, Fliegauf M, Loges NT, Wildhaber J, Noone PG, Kennedy M, Antonarakis SE, Blouin JL, Bartoloni L, Nusslein T, Ahrens P, Griese M, Kuhl H, Sudbrak R, Knowles MR, Reinhardt R, Omran H. DNAH5 mutations are a common cause of primary ciliary dyskinesia with outer dynein arm defects. *Am J Respir Crit Care Med* 2006;**174**:120–6.
16. **Duriez B,** Duquesnoy P, Escudier E, Bridoux A-M, Escalier D, Rayet I, Marcos E, Vojtek A-M, Bercher J-F, Amselem S. A common variant in combination with a nonsense mutation in a member of the thioredoxin family causes primary ciliary dyskinesia. *PNAS* 2007;**104**:3336–41.
17. **Moore A,** Escudier E, Roger G, Tamalet A, Pelosse B, Marlin S, Clement A, Geremek M, Delaisi B, Bridoux AM, Coste A, Witt M, Duriez B, Amselem S. RPGR is mutated in patients with a complex X linked phenotype combining primary ciliary dyskinesia and retinitis pigmentosa. *J Med Genet* 2006;**43**:326–33.
18. **Failly M,** Saitta A, Munoz A, Falconnet E, Rossier C, Santamaria F, de Santi MM, Lazor R, DeLozier-Blanchet CD, Bartoloni L, Blouin JL. DNAI1 mutations explain only 2% of primary ciliary dykinesia. *Respiration* 2008;**76**:198–204.
19. **Omran H,** Haffner K, Volkel A, Kuehr J, Ketelsen UP, Ross UH, Konietzko N, Wienker T, Brandis M, Hildebrandt F. Homozygosity mapping of a gene locus for primary ciliary dyskinesia on chromosome 5p and identification of the heavy dynein chain DNAH5 as a candidate gene. *Am J Respir Cell Mol Biol* 2000;**23**:696–702.
20. **Kispert A,** Petry M, Olbrich H, Volz A, Ketelsen UP, Horvath J, Melkaoui R, Omran H, Zariwala M, Noone PG, Knowles M. Genotype-phenotype correlations in PCD patients carrying DNAH5 mutations. *Thorax* 2003;**58**:552–4.
21. **Tan SY,** Rosenthal J, Zhao XQ, Francis RJ, Chatterjee B, Sabol SL, Linask KL, Bracero L, Connelly PS, Daniels MP, Yu Q, Omran H, Leatherbury L, Lo CW. Heterotaxy and complex structural heart defects in a mutant mouse model of primary ciliary dyskinesia. *J Clin Invest* 2007;**117**:3742–52.
22. **Afzelius BA MB,** Bergstrom SE. Immotile cilia syndrome (primary ciliary dyskinesia), including Kartagener syndrome. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, eds. *The metabolic and molecular basis of inherited disease*, 8th ed. New York: McGraw-Hill Medical Publishing Division, 2001:4817–27.
23. **Afzelius BA.** Genetical and ultrastructural aspects of the immotile-cilia syndrome. *Am J Hum Genet* 1981;**33**:852–64.
24. **Attanasio C,** David A, Neerman-Arbez M. Outcome of donor splice site mutations accounting for congenital afibrinogenemia reflects order of intron removal in the fibrinogen alpha gene (FGA). *Blood* 2003;**101**:1851–6.
25. **King SM.** AAA domains and organization of the dynein motor unit. *J Cell Sci* 2000;**113**:2521–6.
26. **Neuwald AF,** Aravind L, Spouge JL, Koonin EV. AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* 1999;**9**:27–43.
27. **Katsanis N,** Ansley SJ, Badano JL, Eichers ER, Lewis RA, Hoskins BE, Scambler PJ, Davidson WS, Beales PL, Lupski JR. Triallelic inheritance in Bardet-Biedl syndrome, a Mendelian recessive disorder. *Science* 2001;**293**:2256–9.
28. **Chilvers MA,** Rutman A, O'Callaghan C. Ciliary beat pattern is associated with specific ultrastructural defects in primary ciliary dyskinesia. *J Allergy Clin Immunol* 2003;**112**:518–24.
29. **Noone PG,** Leigh MW, Sannuti A, Minnix SL, Carson JL, Hazucha M, Zariwala MA, Knowles MR. Primary ciliary dyskinesia: diagnostic and phenotypic features. *Am J Respir Crit Care Med* 2004;**169**:459–67.